Proteomic Approaches to Studying Drug Targets and Resistance in *Plasmodium*

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Abstract: Ever increasing drug resistance by *Plasmodium falciparum*, the most virulent of human malaria parasites, is creating new challenges in malaria chemotherapy. The entire genome sequences of *P. falciparum* and the rodent malaria parasite, *P. yoelii yoelii* are now available. Extensive genome sequence data from other *Plasmodium* species including another important human malaria parasite, *P. vivax* are also available. Powerful research techniques coupled to genomic resources are needed to help identify new drug and vaccine targets against malaria. Applied to *Plasmodium*, proteomics combines high-resolution protein or peptide separation with mass spectrometry and computer software to rapidly identify large numbers of proteins expressed from various stages of parasite development. Proteomic methods can be applied to study sub-cellular localization, cell function, organelle composition, changes in protein expression patterns in response to drug exposure, drug-protein binding and validation of data from genomic annotation and transcript expression studies. Recent high-throughput proteomic approaches have provided a wealth of protein expression data on *P. falciparum*, while smaller-scale studies examining specific drug-related hypotheses are also appearing. Of particular interest is the study of mechanisms of action and resistance of drugs such as the quinolines, whose targets currently may not be predictable from genomic data. Coupling the *Plasmodium* sequence data with bioinformatics, proteomics and RNA transcript expression profiling opens unprecedented opportunities for exploring new malaria control strategies. This review will focus on pharmacological research in malaria and other intracellular parasites using proteomic techniques, emphasizing resources and strategies available for *Plasmodium*.

Key Words: Malaria, Parasite, Protein, Mass Spectrometry, Genomics, Chloroquine, Artemisinin

INTRODUCTION

Research on *Plasmodium falciparum*, a protozoan parasite and causative pathogen of the lethal form of human malaria, has entered the post-genomic phase. Yet the disease continues its devastating course, with recent mortality estimates of 700,000 - 2.7 million deaths annually, the majority of which occur in young children [1]. Parasite resistance to the most affordable antimalarial drugs has severely limited the available therapeutic options for Africa, where falciparum malaria takes it greatest toll [2]. New and inexpensive drugs against malaria are urgently needed in all parts of the world where the disease is endemic [3,4]. Modern genomics-based approaches such as proteomics offer increased hope for the discovery of promising new drug targets by virtue of their ability characterize complex parasite biology and biochemistry.

Proteomics is an increasingly popular approach to address both large and small-scale hypotheses related to microbial pathogens [5,6]. The proteome may be defined as the set of expressed proteins in a cell, tissue or organism at a given point in time. The goal of proteomics is the complete identification and quantification of a specific proteome, with the ultimate goal of revealing protein function as part of a complex, interrelated system. Most proteomic studies involve the high-resolution separation of proteins from a complex protein mixture isolated under certain experimental conditions. All, or certain proteins of interest are identified using MS data linked to a genome sequence database by specialized software. In *P. falciparum*, proteomics has led to a more complete understanding of large numbers of proteins throughout the complex life cycle of the parasite, in both the human and mosquito host.

Sequencing of the *P. falciparum* [7] and *P. yoelii yoelii* [8] genomes has been completed, and large amounts of sequence data from other *Plasmodium* species are also available [9]. Therefore, within the limits of sequence annotation, all encoded drug targets, or biochemical pathways that offer the possibility for exploitation for drug development are contained in these databases. Bioinformatic analyses of this sequence information has already led to the discovery of several putative drug targets, including enzymes from biosynthetic pathways specific to the parasite apicoplast, a possible target of artemisinin, and many new proteases [7,10-13]. The speed with which these data can accelerate drug development is illustrated by the rapid clinical testing of fosmidomycin in field trials less than three years from the identification of the drug target in the genome sequence database [14,15]. However, many more promising drug targets likely exist in the malaria parasite. By necessity, these may be revealed from basic research, as the function of most predicted proteins in *P. falciparum* remain unknown [7].

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The *Plasmodium* genomic sequence alone is inadequate to predict global protein expression patterns within the parasite. Gene expression studies utilizing DNA microarrays have provided detailed pictures of RNA transcript regulation throughout the blood-stages of the parasite life cycle [16,17]. However, the relationship between transcript and protein abundance in *P. falciparum* has yet to be established. In other organisms, transcript abundance is often poorly related to protein expression levels [18,19]. Additionally, transcript data does not provide direct information on cellular localization, post-translational modification states or protein-protein interactions [20]. Only direct studies at the protein level can routinely provide this information.

Proteomic technologies make functional studies of *Plasmodium* proteins at the organelle and whole cell level increasingly feasible. Two recent high-throughput proteomic studies have produced extensive stage-specific protein expression data for *P. falciparum* [21,22]. Investigations using novel proteomic methods to identify antimalarial drug targets have recently been published [23,24]. Despite these reports, the relative scarcity of proteomic studies in malaria are in part due to unique aspects of *Plasmodium* biology that make it both a challenging and attractive organism for such applications. However, numerous studies on bacterial pathogens and other model organisms such as *Saccharomyces cerevisiae* using proteomics illustrate the value of these approaches [6,25]. Proteomic techniques for identifying potential drug targets and mechanisms in *Plasmodium* can be envisioned in at least three ways, including: i) basic biological studies of cell composition, regulation and function; ii) global protein expression changes in response to drug challenge; iii) in specific assays to screen proteins bound to chemical libraries or covalent drug adducts. This review will focus on applications of proteomic techniques to study *Plasmodium* biology, with the goal of revealing potential drug targets as well as mechanisms of action and resistance of current antimalarials. Up to date, complementary reviews on new drug targets in *Plasmodium* appear in this volume and elsewhere [13,26-28].

PROTEOMIC RESOURCES FOR *PLASMODIUM*

Genomic sequence data provides the foundation of modern proteomic studies. Driven by the need for malaria control through vaccine and drug development, the genome of *P. falciparum* clone 3D7 has been completely sequenced through the combined efforts of the Sanger Institute, Stanford University Genome Technology Center and The Institute for Genome Research / Naval Medical Research Center [7]. The genome comprises ~23 million base pairs over 14 chromosomes, with 5,268 predicted genes. Seventy-percent of these genes have been matched to ESTs or expressed proteins. Importantly, about two-thirds of the predicted proteins do not have adequate resemblance to proteins found in other organisms to warrant functional assignment. This indicates that the majority of proteins encoded by the genome are unique to *P. falciparum* (or to the *Plasmodia*), creating both opportunity and challenges for drug discovery. Partial sequence data is currently available for other *Plasmodium* species including *P. vivax, P. yoelii, P. chabaudi, P. knowelsi* and *P. berghei* [29]. With extensive sequence data now available, both large and small-scale proteomic studies in malaria are feasible.

PlasmoDB (see www.PlasmoDB.org for a complete list of available resources), the official data center of the *P. falciparum* genome sequencing effort, has extensive, publicly available proteomic resources [29]. Protein expression data derived from high-throughput proteomic studies of different stages of the *P. falciparum* life cycle is available for detailed analysis and can be validated with expression profiling results from DNA microarray studies (and vice-versa) [16,17,21]. Most importantly, PlasmoDB features download-able files containing the *Plasmodium* genomic sequences, allowing investigators to link their own on-site mass spectrometry software directly to sequence data. The site also contains web-based software for both the *de novo* sequence interpretation of CID mass spectral data and for identifying proteins based on their peptide mass fingerprints (e.g. MALDI data). The MS searches are interfaced directly with the *Plasmodium* genomic data. To assist in categorizing proteins based on recognized motif and functional domains, Gene Ontology (GO) assignments are also provided. As additional functional genomics data are generated, they will also be incorporated and integrated into the PlasmoDB database. RNA transcript profile databases derived from expression studies of the *P. falciparum* life cycle are available for download at http://malaria.ucsf.edu/ and http://carrier.gnf.org/publications/CellCycle/ [16,17]. Known biochemical pathways of the malaria parasite have been assembled at http://sites.huji.ac.il/malaria/.

PROTEOMIC APPROACHES IN MALARIA RESEARCH

An ever-increasing array of methods are available for proteomic studies, many of which are beyond the scope of this review. See [5,30,31] for detailed reviews on state-of-the-art proteomic techniques.

Two-Dimensional Gel Electrophoresis

Undoubtedly the most commonly used method in proteomic investigations is 2DE [32]. To separate proteins from complex mixtures, 2DE relies on isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension (see Fig. (**1**)) [33,34]. The combination of the two orthogonal separation techniques has the theoretical capacity to resolve thousands of proteins [35]. Typically, individual protein spots from a stained gel are cut out by hand, subjected to specific proteolytic digestion proceeded by an extraction step to elute the resultant peptides. To identify the protein, experimentally derived MS data from the peptide mixture must be linked with the genome sequence database of interest using specialized software such as SEQUEST [36]. Different types of MS are amendable to 2DE-based experiments, including MALDI-TOF and LC-MS/MS (see [37] for a review on MS for proteomic applications). Proteins are identified by virtue of their proteolytic mass fingerprints, which can be matched to theoretical "*in silico*" mass fingerprints of proteins in a database [38,39]. Major limitations of 2DE include the inability to resolve low-

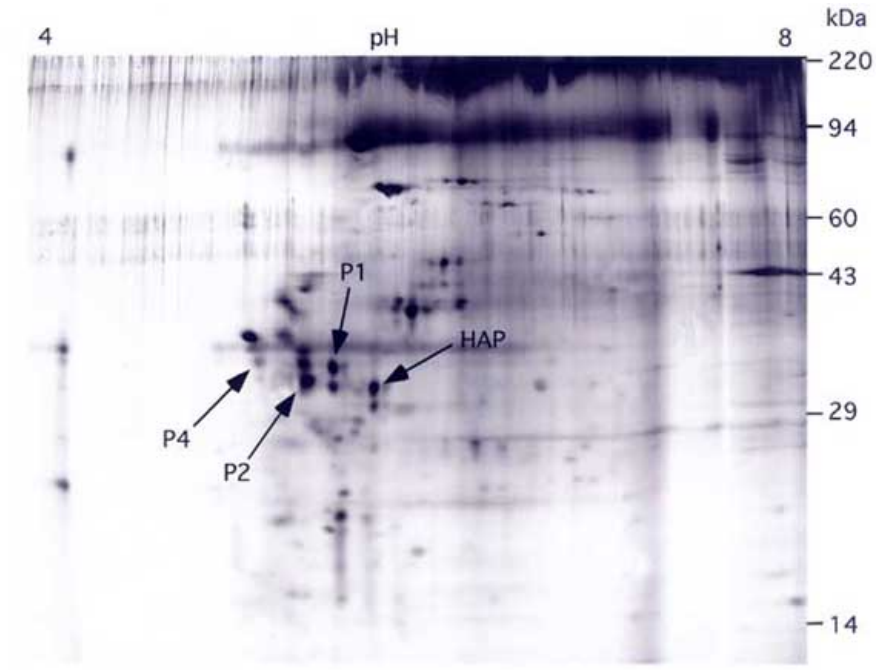

Fig. (1). 2D gel showing proteins from isolated food vacuoles of *P. falciparum* [71]. Arrows point to protein spots identified by MS as plasmepsin 1 (P1), plasmepsin 2 (P2), plasmepsin 4 (P4) and histo-aspartic protease (HAP). The plasmepsins and plasmepsin-like enzymes are important food vacuole hemoglobinases and potential antimalarial drug targets [46].

abundance proteins, those with large numbers of transmembrane domains, of large molecular mass or extreme isoelectric point. Despite these limitations, 2DE remains an important resource, and this trend is expected to continue, especially as technology in the field improves [32]. 2DE is accessible and affordable to individual laboratories and has the inherent appeal of creating a visual display of experimental data [40].

2DE has been used for selective proteome analysis of *Plasmodium* and other intracellular human parasites. Intracellular blood stages of *P. falciparum* extensively modify the host cell by exporting their own proteins to the erythrocyte membrane. These proteins appear to function in nutrient acquisition, cytoadherence and immune evasion, and thus are potentially important drug and vaccine targets [41-43]. In order to identify some of these membrane proteins, Rabilloud *et al.* used 2DE to compare proteomes from *P. falciparum*-infected and uninfected erythrocyte ghosts [44]. Many proteins were resolved that were believed to be of parasite origin, although they were not identified due to the paucity of *P. falciparum* genomic sequence available at the time. As this study focused on membrane proteins, the promise of better solubilizing agents for hydrophobic proteins was demonstrated. Cohen *et al.* used 2DE to resolve more than 1000 proteins from cultured tachyzoite stage parasites of *T. gondii* [45]. Studies indicated that using narrower pH ranges could resolve up to 4000 individual proteins. Despite known limitations of 2DE, this study clearly illustrates that large number of proteins that can be studied by this well-established method of protein separation.

Because 2DE creates a visual array of proteins, it is ideally suited for detecting changes in protein abundance and post-translational states following gene transfection or knockout of potential drug targets. In *P. falciparum*, the food vacuole contains several related aspartic proteases known as the plasmepsins. These enzymes are specialized in hemoglobin digestion and are being extensively investigated as drug targets [46,47]. In plasmepsin 4 gene knockout studies, 2DE is proving useful to detect the disappearance of the protein from the parasite food vacuole (J. B. Dame, personal communication). To investigate mechanisms of antifolate resistance in another parasite model, Drummelsmith *et al*. [48] used multiple 2D gels with overlapping pH ranges to resolve ~3700 proteins from *Leishmania major*. Pteridine reductase PTR1, identified by MALDI-TOF from the excised protein spot, was overexpressed in a cultured methotrexate-resistant mutant line of *L. major*. PTR1 overexpression is known to be a primary cause of methotrexate resistance in *L. major*, supporting the hypothesis that exposure to a drug can cause detectable changes in protein levels related to its mechanism of action or resistance. Additionally, a transfected line of *L. major* overexpressing the trypanothione reductase gene produced up to a 4-fold increase in several different post-translationally modified forms of the protein that were easily observable on 2DE [48].

High Throughput Proteomics

Because of the drawbacks of 2DE, studies attempting to describe large proteomes should use a combination of methods. In the last several years, a high throughput

technique for identifying thousands of proteins from complex mixtures has emerged [36]. This system, termed MudPIT, relies on the separation of peptides by two-phase liquid microchromatography coupled directly to a tandem mass spectrometer (see Fig. (**2**)). Rather than 2D gels, MudPIT utilizes a microcapillary column (i.d. <100 μm) packed with a strong cation exchange resin followed by a C18 reverse-phase matrix for high-resolution peptide separation. Peptides from a protease-digested protein sample, such as a soluble or membrane fraction of a cell lysate, are eluted by salt and organic solvent gradients directly into a tandem mass spectrometer where they are fragmented to produce a spectrum characteristic of the amino acid sequence [49]. Using the SEQUEST algorithm to identify peptides, experimental fragmentation patterns derived by the tandem mass spectra are matched to theoretical fragmentation patterns derived from proteins in a database [36]. A great advantage of gel-free systems such as MudPIT is an improved ability to detect hydrophobic and low abundance proteins as well as the ability to conduct "iterative" database queries for post-translational modifications [25,50]. Large-scale proteomic endeavors using MudPIT include the identification of thousands of different proteins from *S. cerevisiae* and the rice plant, *Oryza sativa*, including membrane proteins, low abundance proteins and those of extreme PI [25,51].

In a landmark study, Florens and colleagues characterized and compared the protein complement of four stages of the *P. falciparum* life cycle: sporozoites, merozoites, trophozoites and gametocytes using the MudPIT system [21]. Remarkably, they detected 46% of all predicted gene products, corresponding to 2415 different parasite proteins. Many proteins were unique to each life cycle stage, while only 152 were common to all four stages, suggesting cohesive regulation of protein subsets involved in stage-specific functions. At the chromosomal level, 168 clusters were identified containing three to six consecutive, co-expressed genes. Only 67 of these clusters had two or more genes with ascribed functional annotation, yet 30 contained multiple genes of clearly related function. Thus, putative function may be ascribed to other proteins whose gene locus resides within a functional cluster. The sensitivity of the MudPIT approach is illustrated by: i) the characterization of 513 unique proteins from poorly accessible stages such as the sporozoite, and ii) the detection of 439 proteins containing at least one predicted transmembrane segment. Detection of membrane proteins in proteomic studies is noteworthy, as their hydrophobicity usually precludes their analysis by 2DE methods.

In another large-scale study, Lasonder *et al*. used both a gel and gel-free approach to identify 1289 proteins from trophozoites/schizonts, gametocytes and gametes, representing about 23% of the predicted protein set from *P. falciparum* [22]. Soluble and insoluble protein fractions from each stage were separated by single-dimension SDS-PAGE. Uniform gel slices were cut out and the assorted proteins

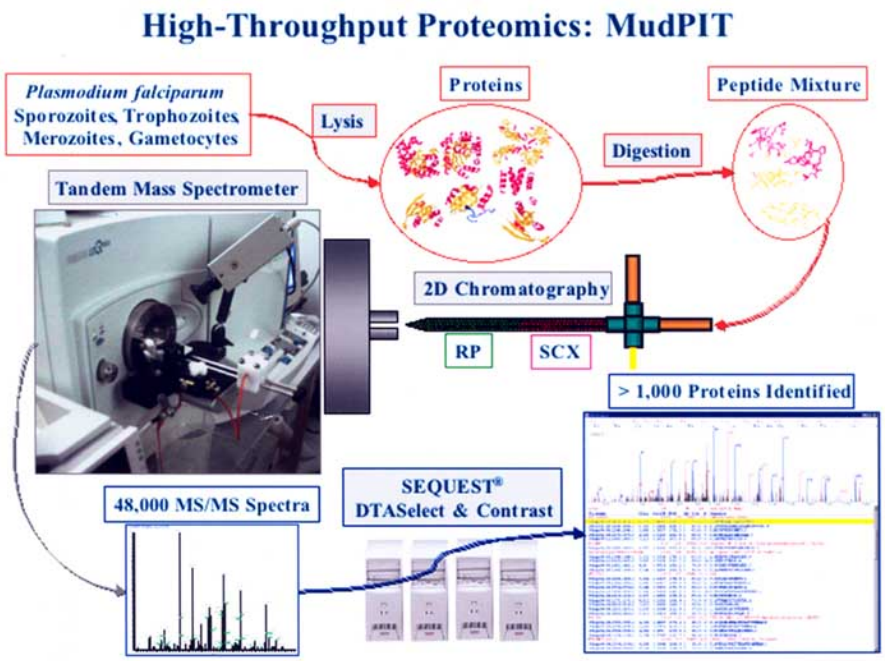

Fig. (2). Schematic illustrating the high-throughput proteomic analysis of large numbers of *Plasmodium* proteins by MudPIT. Complex protein mixtures are digested with trypsin and injected into a two-phase micro-capillary HPLC column. Peptides are sequentially eluted from a strong cation exchange (SCX) phase, followed by reverse-phase (RP) separation and eluted directly into a tandem mass spectrometer. Peptide identities are determined by the SEQUEST algorithm through the correlation of the mass spectra of fragmented peptides to a sequence database. For further details, see [21].

contained in the matrix were subjected to in-gel digestion with trypsin. Insoluble protein fractions were also differentially extracted without prior gel separation and proteolytically digested. Peptide mixtures were then analyzed by NanoLC-MS/MS. Similar to the latter study, proteins subsets were identified that were unique to each stage. Additionally, several proteins were detected that were not in the original annotation of the genome. Although not as sensitive as MudPIT, a gel-based system offers the advantage of providing estimates on protein masses that help to validate matches of peptides to their corresponding proteins by establishing a correlation between the actual and predicted masses from gene annotations [22]. Both high throughput studies have provided data supporting the hypothesis that *P. falciparum* has carefully-ordered, stage-specific subsets of genetic information, in agreement with results from large-scale transcript profiling studies [16,17].
Chemical Proteomics.

Chemical proteomics (also called functional proteomics), or proteome mining, utilizes MS to identify proteins bound to ligands in screening assays designed to search for potential drug targets [30,52]. Fluorescently or isotopically labeled chemical probes, or those linked to solid support matrices, are used to bind specific proteins from whole cell or fractionated lysates. In this way, large libraries of simple chemicals based on motifs of natural ligands or known drug structures are screened for binding specificity and selectivity against whole proteomes. Following identification of putative drug targets or selective inhibitors of specific enzymes, the next challenge will be the verification of efficacy in living cells.

In malaria parasites, cysteine proteases serve multiple functions, and are attractive drug targets [53]. Greenbaum *et al.* used an MS approach to screen *Plasmodium* proteins bound to small chemical probes designed to mimic cysteine protease inhibitors [24]. These highly specific probes are electrophilic and will covalently bind to their target molecules [54]. Blood stages of *P. falciparum* were exposed to a fluorescent-labeled probe based on the peptide epoxide of E64 to characterize the major cysteine proteases. Affinity labeled proteins were separated by SDS-PAGE, subjected to in-gel tryptic digestion, and identified by MS with database searching. Among the several cysteine proteases identified, the results showed that only falcipain-1 was active in the invasive merozoite stage. Importantly, selective inhibition of falcipain-1 blocked parasite invasion of erythrocytes, validating this protein as a drug target. This strategy can be used to both identify potential drug targets and to screen chemical libraries for selectivity within a particular family of proteins.

Chloroquine-protein binding associated with drug efficacy in *P. falciparum* has yet to be established. Graves *et al*. exploited the chemical similarity between the quinoline nucleus and the purine structure of ATP to identify quinoline binding proteins from *P. falciparum* and human erythrocyte preparations [23]. Cell lysates were first passed over an ATP-sepharose column to select for purine binding proteins. Bound proteins that could be displaced by chloroquine, mefloquine or primaquine were identified by MS. Two host erythrocyte proteins that specifically bound chloroquine were quinone reductase 2 and aldehyde dehydrogenase 1. Although these are novel human protein targets of chloroquine, their role in the antimalarial mechanism of quinolines, if any, is unknown. Consistent with heme binding as a basis of the antimalarial activity of quinolines [55], no *P. falciparum* proteins were found that bind to chloroquine under the assay conditions. Nonetheless, further developments in chemical screening techniques will ultimately provide important information on drug targets and mechanisms in *Plasmodium*.

Quantitative Proteomics

An important goal of many proteomic studies is the relative quantification of proteins between a control and perturbed sample. Quantification is a developing aspect of proteomics, but challenges remain [56]. For 2DE, protein staining has some value to produce quantitative results, but is limited by the dynamic range of the chosen stain. Silver staining, with a dynamic range limited to a single order of magnitude, also interferes with the MS analysis unless a less sensitive, glutaraldehyde-free staining procedure is used [57]. Coomassie Blue staining, while compatible with MS, loses some of the sensitivity that many experimental conditions demand. The recent development of fluorescent proteins dyes such as the SPYRO series give superior results in terms of sensitivity, quantification and ease of use compared to traditional colloidal Coomassie Blue and silver stains, and are compatible with mass spectrometry [40,58]. SYPRO Orange and Ruby staining was recently used to detect ~3700 protein spots from the *L. major* proteome by 2DE [48]. SYPRO Ruby for instance, a ruthenium-based stain, can indicate differences in relative protein quantity from 2 to 1000-fold, greatly increasing the information derived from 2DE.

MS can be used to determine relative differences in protein quantity between biological samples through differential metabolic labeling with an essential amino acid or affinity label incorporating a non-radioactive, heavy isotope [59,60]. Since a labeled peptide will have a different mass compared to the identical unlabeled peptide, they can be distinguished by their mass spectra shift, while relative abundance is determined by the ratio of their mass peak intensities [60,61]. Deuterated isoleucine (Ile-d3) may be an ideal metabolic label for *P. falciparum* grown by *in vitro* culture because it is absent from hemoglobin (the parasite's primary amino acid source), yet is abundant in most parasite proteins due to the (A+T)-rich codon bias of *P. falciparum* DNA [62]. The unique mass of labeled isoleucine has the additional benefit of being distinguishable from leucine, adding statistical power to peptide matches made by SEQUEST. Isotope incorporation must be essentially 100% to be accurate. This may be achievable using Ile-d3 in *P. falciparum* culturing, as the parasites do not synthesize isoleucine, and a serum substitute (Albumax; Invitrogen Corporation) without free amino acids is used for culturing. Thus, the parasites should have no other source of isoleucine except for what is supplied exogenously. Because labeled and unlabeled samples are analyzed simultaneously by MS, a further advantage of heavy isotope labeling is that samples are mixed prior to work-up, eliminating variation due to the

preparation steps. Differential metabolic labeling was recently used in combination with MudPIT for the quantitative analysis of over 800 proteins from the *S. cerevisiae* proteome, demonstrating the effectiveness of the method in a large-scale approach [61]. Metabolic and affinity labeling methods are applicable to both gel and gel-free proteomic approaches, and will be valuable for assessing quantitative protein changes in *Plasmodium* following drug exposure.

CHALLENGES AND ADVANTAGES OF *PLASMODIUM* IN PROTEOMIC STUDIES

The human *Plasmodia* have a complex life cycle that compounds the difficulty in developing antimalarial drugs and vaccines. For example, the pre-erythrocytic hepatic stage of *Plasmodium* is clearly an important stage for drug targeting, yet technical difficulties impede obtaining sufficient quantities of pure or enriched material for efficient drug screening or biological studies [63]. Access to liver stage parasites would be particularly valuable, as the mechanism of action of drugs that target these stages, such as primaquine and tafenoquine, are not understood [28]. Most antimalarial drugs are primarily effective against intraerythrocytic stages of *P. falciparum*. In contrast, blood stages, including the sexually differentiated gametocytes, are readily accessible because they can be easily cultured *in vitro* [64,65]. Thus, large quantities of parasites can be exposed to various drugs and subsequently purified for proteomic studies. Unfortunately, other human malaria parasite species cannot yet be cultured *in vitro*.

Characterizing parasite organelle proteomes will help in the study of their biogenesis and function, as well as elucidate entire biochemical pathways to exploit for drug development. Huang *et al.* used 2DE and a tandem MALDI-TOF MS approach to identify all the major proteins of purified 20S proteasomes from cultured, extracellular forms of *Trypanosoma brucei*, the causative agent of the African trypanosomiases [66]. However, intracellular stages of apicomplexan parasites like *Plasmodium* and *T. gondii* reside within two compartments delimited by the host cell membrane and the parasitophorous vacuolar membrane, increasing the challenges of isolating subcellular components [67,68]. Owing to the presence of iron-containing heme, *Plasmodium* food vacuoles are heavy and can be isolated to high purity by cell fractionation and differential centrifugation [69,70]. Dame *et al.* have taken advantage of this method to verify the localization of four aspartic hemoglobinases to the food vacuole using 2DE (see Fig. (**1**)) [71]. Proteomic analysis of carefully isolated organelle prepar-ations may yield many surprises. For example, Akompong *et al*. have shown that the *P. falciparum* food vacuole contains membrane complexes derived from the endoplasmic reticulum [72]. Some success has also been achieved in isolating apical organelles from *Plasmodium* and other Apicomplexa [68]. The apical organelles play a critical role in cellular invasion, and a detailed characterization of their proteome would be important for development of invasion inhibiting drugs. A lack of strategies for the isolation of other *Plasmodium* organelles represents an important bottleneck in the proteomic study of malaria parasites.

Genetic modification through stable transfection in *Plasmodium*, while difficult, is becoming increasingly routine. Various transfection strategies have been developed for *P. falciparum* [73-75], for the rodent malaria parasites, *P. berghei* [76,77] and *P. yoelii* [78] and the primate malaria, *P. knowlesi* [79]. For pharmacological studies, transfection is invaluable because it allows the study of phenotypic response resulting from single amino acid changes or substitution of entire alleles related to drug response or resistance. In response to modifications to potential drug target proteins of unknown function, expression levels of proteins in related biochemical pathways may also change. In *P. falciparum*, mutations in codon 76 of the *pfcrt* gene protein that confer chloroquine resistance also alter intercompartmental pH [80,81]. Some of these mutations differentially affect parasite fitness, manifested through decreased culture expansion rates [R. Cooper, unpublished data]. In this case, comparing proteomes of mutant lines may reveal differences induced by compensatory mechanisms that might give clues to PfCRT function. Global proteomic analysis of changes associated with drug resistance mutations in *Plasmodium* or other microbial pathogens allows for discovery without expectations that can bias experimental methods away from capturing potentially important information [6].

Another advantage transfection brings to proteome analysis is that genetically modified parasites of the same strain can be compared. While it is tempting to compare specific proteomes between chloroquine-sensitive and chloroquine-resistant field isolates, it is impossible to control for genetic background. Parasite proteome content may be variable due to clonal differences that are detectable on 2DE [82] that could further complicate the identification of changes directly associated with the drug resistance phenomena.

VALIDATION OF DRUG AND VACCINE TARGETS

Proteomics has the capacity to both verify and complement data from other genomic techniques, including gene annotation, mRNA transcript profiling and quantitative trait loci mapping. Gene structure may be determined through their corresponding protein products, whereas genes may have been missed in the bioinformatic analysis of a complete genomic sequence. Six proteins detected on 2DE from a *Mycobacterium tuberculosis* proteome were not previously predicted from the *M. tuberculosis* genome database by sequence annotation [83]. The highly (A+T)-rich genome of *P. falciparum* creates difficulties in the prediction of open reading frames from the sequence data. More than 100 peptides were identified by mass spectrometry from a *P. falciparum* proteome that were not predicted in the early annotations of the genome [22]. Analysis of these peptides led to the re-annotation of the corresponding gene structure and new GO term assignments. This emphasizes the importance of searching MS data not only among predicted proteins, but also against the whole genome database.

Only about 35% of the proteins encoded by the *P. falciparum* genome are of known function [7]. Therefore, an important goal of protein and transcript expression studies is to assign putative function to uncharacterized genes. Florens *et al*. first demonstrated the presence of expression clusters

in *P. falciparum* at the protein level, suggesting that these clusters may represent a means to identify functions of previously hypothetical proteins [21]. It has since been shown in *P. falciparum* that genes with similar function frequently have similar transcript profiles [16,17]. Therefore, uncharacterized proteins may be cross-referenced to the transcript expression databases to make an inference about cellular function based on transcript grouping into expression clusters. Detailed expression and cellular localization profiles will also verify that drug or vaccine targets are expressed during the appropriate phase of the parasite life cycle for implementation of disease control strategies.

The correlation between mRNA levels and protein abundance in *P. falciparum* has yet to be characterized in detail. Studies in the yeast *S. cerevisiae* have reported somewhat conflicting results regarding mRNA versus protein abundance, ranging from low to good correlations [19,84]. Preliminary analysis of a small number of genes from *P. falciparum* has indicated a positive correlation between transcript levels and protein abundance [22]. Parallel quantitative proteomic studies will therefore be helpful for interpreting results from mRNA expression studies where transcript levels may be altered following drug exposure [85]. While increases or decreases in relative protein abundance likely proceed changes in transcript levels from drug-exposed parasites, the magnitude of these changes are unknown.

PROTEOMICS OF THE ARTEMISININS

The artemisinins are unique antimalarials because of their ability to form reactive intermediates and alkylate multiple proteins [86]. Thus they make attractive subjects for proteomic studies because not all targets of artemisinin are likely to be predicted from the genome database.

Artemisinin (qinghaosu) is an endoperoxide-containing sesquiterpene lactone, Fig. (**3**), isolated from sweet wormwood, *Artemisia annua*. Several semi-synthetic and synthetic derivatives of artemisinin are currently being developed or are actively being used to treat malaria, primarily as part of drug combination therapies [4,87,88]. Artemisinin derivatives have cured millions of cases of malaria, are active against all drug-resistant parasite strains and are evolving into a worldwide mainstay in the antimalarial arsenal [86,89]. The precise mechanism of action of artemisinin remains unclear and controversial [90-94]. Much evidence points to Fe(II)-catalyzed reductive cleavage of the endoperoxide bridge within the parasite, leading to an unstable carbon-centered radical capable of forming protein adducts [86,95]. Under *in vitro* conditions and possibly within the parasite, artemisinin can alkylate heme, but whether this product is important in parasite killing is also debated [94,96-98]. An alternative mechanism put forth suggests that heterolytic opening of the endoperoxide moiety of artemisinin yields a hydroperoxide, leading to a reactive carbonium ion intermediate as well as the formation of hydoxyl radicals [99]. This pathway would also promote alkylation of protein nucleophiles by structurally different intermediates than a carbon-centered free radical derivative [90].

The artemisinins clearly bind to parasite proteins, but a direct link between alkylation and the mechanism of action has yet to be established [86]. Several labeled proteins have been detected from malaria parasites exposed to [^{3}H]-artemisinin derivatives, one of which was subsequently identified as *P. falciparum* translationally-controlled tumor protein (TCTP) [100,101]. The role of TCTP binding in parasite killing is unknown. Recent evidence suggests that artemisinin inhibits the *P. falciparum* sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) orthologue PfATP6, both in the parasite and when expressed in *Xenopus* oocytes [12]. Although direct evidence of binding was not demonstrated, inactivation of PfATP6 by artemisinin was competitive with thapsigargin, a known SERCA inhibitor [102]. Because of their potent alkylating capacity, it is unlikely that the artemisinins have a single, specific protein target entirely responsible for their efficacy. If this was the case, one might expect resistance to arise quickly, characteristic other enzyme-targeting antimalarial drugs such as folate inhibitors and atovaquone [103,104]. Currently, there is no documented clinical resistance to the artemisinins by *P. falciparum*, although it will probably arise given the rapid expansion of its use.

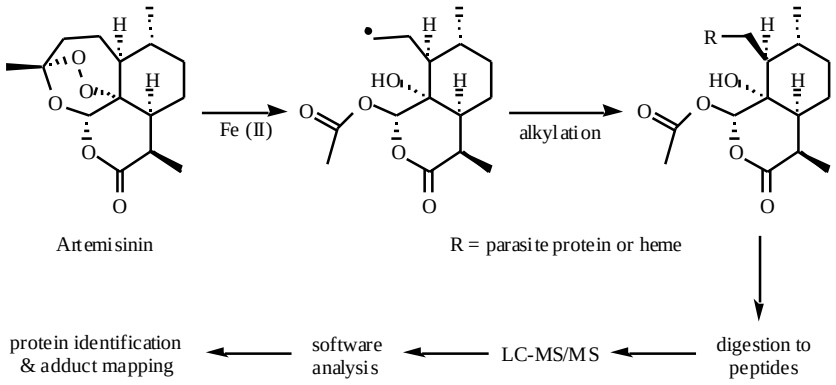

Fig. (3). Proposed pathway of bioactivation of the endoperoxide antimalarial drug, artemisinin. Reductive scission of the peroxy bond by ferrous iron ultimately generates a C4 free radical derivative [95] that can form covalent adducts with parasite proteins and heme [86]. LC-MS/MS and specialized software can identify the alkylated protein and map the artemisinin adduct to a specific amino acid [36,129].

Protein alkylation resulting from exposure to reactive drug intermediates or metabolites is a well-known phenomenon [105,106]. Electrospray-ionization MS has been used to determine the presence of artemisinin adducts on human albumin from an *in vitro* assay [107]. Recently, multiple artemisinin-adducts corresponding to a molecular mass of 282.2 (see Fig (**3**)) were detected from *in vitro* alkylation of microperoxidase-11, a heme undecapeptide, supporting the presence of a C4-centered free radical intermediate under the studied reaction conditions [97]. Combined with LC-MS/MS and SEQUEST, advances in mass spectrometry analysis software such as SALSA, allow not only the detection of drug-protein adducts from biological samples, but mapping of the adducts to the specific alkylated amino acid residues [108]. An advantage of SALSA is that an *a priori* knowledge of precise adduct structure is unnecessary [109]. This is important when the mechanism of reactive intermediate formation is unclear or when there is potential for the modification of adduct structure during sample preparation. Applied to *Plasmodium*, PfATP6 expressed in *Xenopus* oocytes could be labeled by exposure to an [^{3}H]-artemisinin derivative and subsequently isolated by single-dimension SDS-PAGE. The excised protein could tryptically digested, subjected LC-MS/MS and identified using SEQUEST. Additional screening of the mass spectra with SALSA may identify specific amino acid residues containing a covalent artemisinin adduct. Resolving the precise chemical nature of artemisinin-protein adducts will provide important information regarding its pathway of bioactivation within the malaria parasite, as well as potential markers of drug efficacy.

A 2DE approach might also be valuable in several ways to study artemisinin. Lysates prepared from parasites exposed to [^{3}H]-artemisinin are known to show multiple labeled bands on single-dimension SDS-PAGE [100]. The high-resolution capacity of 2DE may resolve proteins not previously detected. However, the utility of 2DE in resolving artemisinin-bound proteins remains to be seen, as most of the proteins alkylated appear to be hydrophobic.

In a study of artemisinin resistance, a 2.5-fold increase in TCTP expression was observed in resistant *P. yoelii* [110]. The effects of the artemisinins on the expression levels of the *P. falciparum* SERCA homolog or other proteins are unknown. In other cell types, thapsigargin selected for concurrent changes in expression levels of SERCA or amino acid changes within the protein [111,112]. 2DE may therefore be valuable in comparing proteomes between control and artemisinin pressured parasites, especially in combination with another strategy such as transcript profiling. Additionally, the analysis of synergistic artemisinin drug combinations, such as mefloquine-artemisinin [113,114], may demonstrate changes in protein expression not seen when parasites are exposed to either drug alone. Proteomics should also be useful in studying artemisinin resistance in the rodent malarias, *P. yoelii,* or *P. berghei*, even if correlation to human malarias has yet to be established. In these models, the artemisinin resistance phenotype is readily induced and is rapidly reversible, suggesting that induction of protein expression, rather than specific mutations in parasite genes may be responsible for resistance [110,115].

CHLOROQUINE

Chloroquine remains a widely used antimalarial, despite extensive therapeutic failures due to drug resistance [103]. The probable molecular target of chloroquine is heme (ferriprotoporphyrin IX), a toxic metabolite released as a result of proteolytic hemoglobin digestion in the parasite food vacuole [116,117]. Chloroquine and other quinolines accumulate to high concentrations in the acid food vacuole because of their weak base properties. They apparently act by non-covalently binding to the soluble heme molecule and inhibiting its sequestration into a harmless microcrystalline form known as hemozoin, or malaria pigment [118-120]. Resistance to chloroquine is a result of amino acid changes in the PfCRT protein, a putative transporter located in the food vacuole membrane [80,121,122]. Subsequent to these mutations, resistant parasites demonstrate a greatly reduced capacity to accumulate chloroquine [80]. The fact that the target for chloroquine is a non-protein, host-derived molecule has implications for proteomic studies. In fact, the mechanism of parasite killing by the quinolines is still not clear. Drug-hematin complexes are believed to harm the parasite by oxidative processes [123]. Thus, cell death may occur through a variety of pathways, rather than by inhibition of a single, specific cellular target. Proteomics can help define global expression changes following exposure to the quinoline drugs, whose mechanisms of action can not currently be predicted from bioinformatic analysis of genome sequence data.

Mutations in the *pfmdr1* gene are associated with the quantitative quinoline response *in vitro*, but direct involvement in resistance has not been demonstrated [121,124]. Exposing cultured *P. falciparum* parasites to chloroquine and the related quinolines, quinine and mefloquine produces an increase in RNA transcript levels of the *pfmdr1* gene [125]. The mechanistically unrelated folate inhibitor pyrimethamine did not induce increases in transcript levels of *pfmdr1*, demonstrating specificity for a gene whose protein product is localized to the food vacuole membrane, where the quinolines are believed to exert their effect [126]. In the chloroquine sensitive 3D7 line of *P. falciparum*, global expression profiling by SAGE following chloroquine exposure indicated significant changes in 123 transcripts [85]. Of course, the effects on actual proteins levels following drug exposure remains to be seen, and will be determined with proteomic studies. As demonstrated, profiling studies at either the transcript and protein level will need to be conducted with multiple drugs, of both related and unrelated mechanisms [85]. This will help differentiate those transcripts whose abundance changes are more likely related to specific drug mechanisms as opposed to general toxic responses. Further studies are required to establish if a characteristic “drug fingerprint” of expressional changes occurs with quinoline exposure in general.

How the expression level changes of 123 transcripts are related to the mechanism of action of chloroquine remains to be sorted out. However, there is considerable evidence that

exposure to certain drugs will cause changes in proteins physiologically related to the mechanism of the drug. Exposure of cultured *P. falciparum* to geldanamycin, an inhibitor of the Hsp90 protein, caused an increase in abundance of both PfHsp90 and PfHsp70 [127]. Isoniazid exposure in a sensitive line of *M. tuberculosis* resulted in induction of gene transcripts in the mycolic acid biosynthetic pathway related to the mechanism of the drug [128].

CONCLUSIONS

The completion of the *P. falciparum* genome ushered in a new phase of malaria research and the potential for improved malaria interventions, drugs and vaccines. Large-scale technologies such as proteomics, combined with sophisticated computational algorithms and databases have already put in the hands of researchers a more clear understanding of this complex human parasite. Yet it remains to be seen precisely how proteomics, alongside other genomic strategies, will aid in the identification of new drug and vaccine targets. As limitations of proteomics continue to diminish as technologies advance, creativity in approaches to investigate new drug targets will be critical in taking advantage of available resources. Although not a panacea, these efforts offer hope that rational new drugs and vaccines are closer to reality.

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ABBREVIATIONS

2DE	=	two-dimensional gel electrophoresis
CID	=	collision induced dissociation
ESTs	=	expressed sequence tags
HPLC	=	high performance liquid chromatography
LC-MS/MS	=	Liquid chromatography - tandem mass spectrometry
MALDI-TOF	=	matrix-assisted laser desorption ionization time-of-flight
MS	=	mass spectrometry
MudPIT	=	multidimensional protein identification technology
PfCRT	=	*Plasmodium falciparum* chloroquine resistance transporter
pfmdr1	=	*Plasmodium falciparum* multiple drug resistance gene 1
SAGE	=	serial analysis of gene expression
SALSA	=	Scoring Algorithm for Spectral Analysis
SDS-PAGE	=	sodium dodecyl sulfate - polyacrylamide gel electrophoresis

REFERENCES

[1] Breman, J. G. *Am. J. Trop. Med. Hyg.*, **2001**, *64*, 1.
[2] Bloland, P. B.; Kachur, S. P.; Williams, H. A. *J. Exp. Biol.*, **2003**, *206*, 3761.
[3] Rosenthal, P. J.; Miller, L. H. In *Antimalarial Chemotherapy. Mechanisms of Action, Resistance and New Direction in Drug Discovery*; Rosenthal, P.J., Ed Humana Press Inc.: Totowa NJ, **2001**, pp 3-13.
[4] Olliaro, P. L.; Taylor, W. R. *J. Exp. Biol.*, **2003**, *206*, 3753.
[5] Hunter, T. C.; Andon, N. L.; Koller, A.; Yates, J. R.; Haynes, P. A. *J. Chromatogr. B*, **2002**, *782*, 165.
[6] Cash, P. Adv. Biochem. Eng. Biotechnol., **2003**, 83, 93.
[7] Gardner, M. J.; Hall, N.; Fung, E.; White, O.; Berriman, M.; Hyman, R. W.; Carlton, J. M.; Pain, A.; Nelson, K. E.; Bowman, S.; Paulsen, I. T.; James, K.; Eisen, J. A.; Rutherford, K.; Salzberg, S. L.; Craig, A.; Kyes, S.; Chan, M. S.; Nene, V.; Shallom, S. J.; Suh, B.; Peterson, J.; Angiuoli, S.; Pertea, M.; Allen, J.; Selengut, J.; Haft, D.; Mather, M. W.; Vaidya, A. B.; Martin, D. M.; Fairlamb, A. H.; Fraunholz, M. J.; Roos, D. S.; Ralph, S. A.; McFadden, G. I.; Cummings, L. M.; Subramanian, G. M.; Mungall, C.; Venter, J. C.; Carucci, D. J.; Hoffman, S. L.; Newbold, C.; Davis, R. W.; Fraser, C. M.; Barrell, B. *Nature*, **2002**, *419*, 498.
[8] Carlton, J. M.; Angiuoli, S. V.; Suh, B. B.; Kooij, T. W.; Pertea, M.; Silva, J. C.; Ermolaeva, M. D.; Allen, J. E.; Selengut, J. D.; Koo, H. L.; Peterson, J. D.; Pop, M.; Kosack, D. S.; Shumway, M. F.; Bidwell, S. L.; Shallom, S. J.; van Aken, S. E.; Riedmuller, S. B.; Feldblyum, T. V.; Cho, J. K.; Quackenbush, J.; Sedegah, M.; Shoaibi, A.; Cummings, L. M.; Florens, L.; Yates, J. R.; Raine, J. D.; Sinden, R. E.; Harris, M. A.; Cunningham, D. A.; Preiser, P. R.; Bergman, L. W.; Vaidya, A. B.; van Lin, L. H.; Janse, C. J.; Waters, A. P.; Smith, H. O.; White, O. R.; Salzberg, S. L.; Venter, J. C.; Fraser, C. M.; Hoffman, S. L.; Gardner, M. J.; Carucci, D. J.; *Nature*, **2002**, *419*, 512.
[9] Carucci, D. J.; Yates 3rd, J. R.; Florens, L. *Int. J. Parasitol.*, **2002**, *32*, 1539.
[10] Wu, Y.; Wang, X.; Liu, X.; Wang, Y. *Genome Res.*, **2003**, *13*, 601.
[11] Seeber, F. *Curr. Drug Targets Immune Endocr. Metabol. Disord.*, **2003**, *3*, 99.
[12] Eckstein-Ludwig, U.; Webb, R. J.; Van Goethem, I. D.; East, J. M.; Lee, A. G.; Kimura, M.; O'Neill, P. M.; Bray, P. G.; Ward, S. A.; Krishna, S. *Nature*, **2003**, *424*, 957.
[13] Cowman, A. F.; Crabb, B. S. *Trends Parasitol.*, **2003**, *19*, 538.
[14] Jomaa, H.; Wiesner, J.; Sanderbrand, S.; Altincicek, B.; Weidemeyer, C.; Hintz, M.; Turbachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E. *Science*, **1999**, *285*, 1573.
[15] Lell, B.; Ruangweerayut, R.; Wiesner, J.; Missinou, M. A.; Schindler, A.; Baranek, T.; Hintz, M.; Hutchinson, D.; Jomaa, H.; Kremsner, P. G. *Antimicrob. Agents. Chemother.*, **2003**, *47*, 735.
[16] Le Roch, K. G.; Zhou, Y.; Blair, P. L.; Grainger, M.; Moch, J. K.; Haynes, J. D.; De La Vega, P.; Holder, A. A.; Batalov, S.; Carucci, D. J.; Winzeler, E. A. *Science*, **2003**, *301*, 1503.
[17] Bozdech, Z.; Llinas, M.; Pulliam, B. L.; Wong, E. D.; Zhu, J.; DeRisi, J. L. *PLoS Biol.*, **2003**, *1*, E5.
[18] Anderson, L.; Seilhamer, J. *Electrophoresis*, **1997**, *18*, 533.
[19] Gygi, S. P.; Rochon, Y.; Franza, B. R.; Aebersold, R. *Mol. Cell. Biol.*, **1999**, *19*, 1720.
[20] Pandey, A.; Mann, M. *Nature*, **2000**, *405*, 837.
[21] Florens, L.; Washburn, M. P.; Raine, J. D.; Anthony, R. M.; Grainger, M.; Haynes, J. D.; Moch, J. K.; Muster, N.; Sacci, J. B.; Tabb, D. L.; Witney, A. A.; Wolters, D.; Wu, Y.; Gardner, M. J.; Holder, A. A.; Sinden, R. E.; Yates, J. R.; Carucci, D. J. *Nature*, **2002**, *419*, 520.
[22] Lasonder, E.; Ishihama, Y.; Andersen, J. S.; Vermunt, A. M.; Pain, A.; Sauerwein, R. W.; Eling, W. M.; Hall, N.; Waters, A. P.; Stunnenberg, H. G.; Mann, M. *Nature*, **2002**, *419*, 537.

[23] Graves, P. R.; Kwiek, J. J.; Fadden, P.; Ray, R.; Hardeman, K.; Coley, A. M.; Foley, M.; Haystead, T. A. *Mol. Pharmacol.*, **2002**, *62*, 1364.

[24] Greenbaum, D. C.; Baruch, A.; Grainger, M.; Bozdech, Z.; Medzihradszky, K. F.; Engel, J.; DeRisi, J.; Holder, A. A.; Bogyo, M. *Science*, **2002**, *298*, 2002.

[25] Washburn, M. P.; Wolters, D.; Yates, J. R. *Nat. Biotechnol.*, **2001**, *19*, 242.

[26] Biagini, G. A.; O'Neill, P. M.; Nzila, A.; Ward, S. A.; Bray. P.G. *Trends Parasitol.*, **2003**, *19*, 479.

[27] Rosenthal, P. J. *J. Exp. Biol.*, **2003**, *206*, 3735.

[28] Wiesner, J.; Ortmann, R.; Jomaa, H.; Schlitzer, M. *Angew. Chem. Int. Ed. Engl.*, **2003**, *42*, 5274.

[29] Bahl, A.; Brunk, B.; Crabtree, J.; Fraunholz, M. J.; Gajria, B.; Grant, G. R.; Ginsburg, H.; Gupta, D.; Kissinger, J. C.; Labo, P.; Li, L.; Mailman, M. D.; Milgram, A. J.; Pearson, D. S.; Roos, D. S.; Schug, J.; Stoeckert, C. J. Jr; Whetzel, P. *Nucleic Acids Res.*, **2003**, *31*, 212.

[30] Graves, P. R.; Haystead, T. A. *Microbiol. Mol. Biol. Rev.*, **2002**, *66*, 39.

[31] Patterson, S. D.; Aebersold, R. H. *Nat. Genet.*, **2003**, *33* Suppl, 311.

[32] Rabilloud, T. *Proteomics*, **2002**, *2*, 3.

[33] MacGillivray, A. J.; Rickwood, D. *Eur. J. Biochem.*, **1974**, *41*, 181.

[34] O'Farrell, P. H. *J. Biol. Chem.*, **1975**, *250*, 4007.

[35] Klose, J.; Kobalz, U. *Electrophoresis*, **1995**, *16*, 1034.

[36] Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. *Nat. Biotechnol.*, **1999**, *17*, 676.

[37] Lin, D.; Tabb, D. L.; Yates, J. R. 3rd. *Biochim. Biophys. Acta*, **2003**, *1646*, 1.

[38] Mann, M.; Hojrup, P.; Roepstorff, P. *Biol. Mass. Spectrom.*, **1993**, *22*, 338.

[39] Griffin, P. R.; MacCoss, M. J.; Eng, J. K.; Blevins, R. A.; Aaronson, J. S.; Yates, J. R. 3rd. *Rapid Commun. Mass. Spectrom.*, **1995**, *9*, 1546.

[40] Patton, W. F.; Beechem, J. M. *Curr. Opin. Chem. Biol.*, **2002**, *6* , 63.

[41] Deitsch, K. W.; Wellems, T. E. *Mol. Biochem. Parasitol.*, **1996**, *76*, 1.

[42] Gero, A. M.; Dunn, C. G.; Brown, D. M.; Pulenthiran, K.; Gorovits, E. L.; Bakos, T.; Weis, A. L. *Curr. Pharm. Des.*, **2003**, *9*, 867.

[43] Mahanty, S.; Saul, A.; Miller, L. H. *J. Exp. Biol.*, **2003**, *206* , 3781.

[44] Rabilloud, T.; Blisnick, T.; Heller, M.; Luche, S.; Aebersold, R.; Lunardi, J.; Braun-Breton, C. *Electrophoresis*, **1999**, *20*, 3603.

[45] Cohen, A. M.; Rumpel, K.; Coombs, G. H.; Wastling, J. M. *Int. J. Parasitol.*, **2002**, *32*, 39.

[46] Coombs, G. H.; Goldberg, D. E.; Klemba, M.; Berry, C.; Kay, J.; Mottram, J. C. *Trends Parasitol.*, **2001**, *17*, 532.

[47] Banerjee, R.; Liu, J.; Beatty, W.; Pelosof, L.; Klemba, M.; Goldberg, D. E. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 990.

[48] Drummelsmith, J.; Brochu, V.; Girard, I.; Messier, N.; Ouellette, M. *Mol. Cell Proteomics*, **2003**, *2*, 146.

[49] Yates, J. R. *Trends Genet.*, **2000**, *16*, 5.

[50] Yates, J. R. 3.; Eng, J. K.; McCormack, A. L.; Schieltz, D. *Anal. Chem.*, **1995**, *67*, 1426.

[51] Koller, A.; Washburn, M. P.; Lange, B. M.; Andon, N. L.; Deciu, C.; Haynes, P. A.; Hays, L.; Schieltz, D.; Ulaszek, R.; Wei, J.; Wolters, D.; Yates, J. R. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 11969.

[52] Adam, G. C.; Sorensen, E. J.; Cravatt, B. F. *Mol. Cell. Proteomics*, **2002**, *1*, 781.

[53] Rosenthal, P. J.; Sijwali, P. S.; Singh, A.; Shenai, B. R. *Curr. Pharm. Des.*, **2002**, *8*, 1659.

[54] Greenbaum, D.; Baruch, A.; Hayrapetian, L.; Darula, Z.; Burlingame, A.; Medzihradszky, K. F.; Bogyo, M. *Mol. Cell. Proteomics*, **2002**, *1*, 60.

[55] Bray, P. G.; Mungthin, M.; Ridley, R. G.; Ward, S. A. *Mol. Pharmacol.*, **1998**, *54*, 170.

[56] Ong, S. E.; Foster, L. J.; Mann, M. *Methods*, **2003**, *29*, 124.

[57] Patton, W. F. *J. Chromatogr. B*, **2002**, *771*, 3.

[58] Lauber, W. M.; Carroll, J. A.; Dufield, D. R.; Kiesel, J. R.; Radabaugh, M. R.; Malone, J. P. *Electrophoresis*, **2001**, *22*, 906.

[59] Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.*, **1999**, *17*, 994.

[60] Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol. Cell. Proteomics*, **2002**, *1*, 376.

[61] Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R. I. *Anal. Chem.*, **2002**, *74*, 1650.

[62] Singer, G. A.; Hickey, D. A. *Mol. Biol. Evol.*, **2000**, *17*, 1581.

[63] Sacci, J. B. Jr.; Azad, A. F. *Int. J. Parasitol.*, **2002**, *32*, 1551.

[64] Trager, W.; Jensen, J. B. *Science*, **1976**, *193*, 673.

[65] Kariuki, M. M.; Kiaira, J. K.; Mulaa, F. K.; Mwangi, J. K.; Wasunna, M. K.; Martin, S. K. *Am. J. Trop. Med. Hyg.*, **1998**, *59*, 505.

[66] Huang, L.; Jacob, R. J.; Pegg, S. C.; Baldwin, M. A.; Wang, C. C.; Burlingame, A. L.; Babbitt, P. C. *J. Biol. Chem.*, **2001**, *276*, 28327.

[67] Lingelbach, K.; Joiner, K. A. *J. Cell. Sci.*, **1998**, *111*, 1467.

[68] Blackman, M. J.; Bannister, L. H. *Mol. Biochem. Parasitol.*, **2001**, *117*, 11.

[69] Goldberg, D. E.; Slater, A. F. G.; Cerami, A.; Henderson, G. B. *Proc. Natl. Acad. Sci.*, **1990**, *87*, 2931.

[70] Saliba, K. J.; Allen, R. J.; Zissis, S.; Bray, P. G.; Ward, S. A.; Kirk, K. *J. Biol. Chem.*, **2003**, *278*, 5605.

[71] Dame, J. B.; Yowell, C. A.; Omara-Opyene, L.; Carlton, J. M.; Cooper, R. A.; Li, T. *Mol. Biochem. Parasitol.*, **2003**, *130*, 1.

[72] Akompong, T.; Kadekoppala, M.; Harrison, T.; Oksman, A.; Goldberg, D. E.; Fujioka, H.; Samuel, B. U.; Sullivan, D.; Haldar, K. *J. Biol. Chem.*, **2002**, *277*, 28923.

[73] Deitsch, K.; Driskill, C.; Wellems, T. *Nucleic Acids Res.*, **2001**, *29*, 850.

[74] Crabb, B. S. *Drug Resist. Update*, **2002**, *5*, 126.

[75] Gardiner, D. L.; Skinner-Adams, T. S.; Spielmann, T.; Trenholme, K. R. *Trends Parasitol.*, **2003**, *19*, 381.

[76] Janse, C. J.; Waters, A. P. *Methods Mol. Med.*, **2002**, *72*, 305.

[77] Thathy, V.; Menard, R. *Methods Mol. Med.*, **2002**, *72*, 317.

[78] Mota, M. M.; Thathy, V.; Nussenzweig, R. S.; Nussenzweig, V. *Mol. Biochem. Parasitol.*, **2001**, *113*, 271.

[79] Kocken, C. H.; Ozwara, H.; van der Wel, A.; Beetsma, A. L.; Mwenda, J. M.; Thomas, A. W. *Infect. Immun.*, **2002**, *70*, 655.

[80] Cooper, R. A.; Ferdig, M. T.; Su, X. Z.; Ursos, L. M.; Mu, J.; Nomura, T.; Fujioka, H.; Fidock, D. A.; Roepe, P. D.; Wellems, T. E. *Mol. Pharmacol.*, **2002**, *61*, 35.

[81] Bennett, T. N.; Kosar, A. D.; Ursos, L. M.; Dzekunov, S.; Singh Sidhu, A. B.; Fidock, D. A.; Roepe, P. D. *Mol. Biochem. Parasitol.*, **2004**, *133* , 99.

[82] Babiker, H. A.; Creasey, A. M.; Fenton, B.; Bayoumi, R. A.; Arnot, D. E.; Walliker, D. *Trans. R .Soc. Trop. Med. Hyg.*, **1991**, *85*, 572.

[83] Jungblut, P. R.; Muller, E. C.; Mattow, J.; Kaufmann, S. H. *Infect. Immun.*, **2001**, *69*, 5905.

[84] Futcher, B.; Latter, G. I.; Monardo, P.; McLaughlin, C. S.; Garrels, J. I. *Mol. Cell. Biol.*, **1999**, *19*, 7357.

[85] Gunasekera, A. M.; Patankar, S.; Schug, J.; Eisen, G.; Wirth, D. F. *Mol. Microbiol.*, **2003**, *50*, 1229.

[86] Meshnick, S. R. *Int. J. Parasitol.*, **2002**, *32*, 1655.

[87] Nosten, F.; Brasseur, P. *Drugs*, **2002**, *62*, 1315.

[88] Borstnik, K.; Paik, I. H.; Posner, G. H. *Mini Rev. Med. Chem.*, **2002**, *2*, 573.

[89] White, N. J.; Olliaro, P. *Med. Trop. (Mars)*, **1998**, *58*, 54.

[90] Olliaro, P. L.; Haynes, R. K.; Meunier, B.; Yuthavong, Y. *Trends Parasitol.*, **2001**, *17*, 122.

[91] Posner, G. P.; Meshnick, S. R. *Trends Parasitol.*, **2001**, *17*, 266.

[92] Jefford, C. W. *Curr. Med. Chem.*, **2001**, *8*, 1803.

[93] Ridley, R. G. *Nature*, **2003**, *424*, 887.

[94] Meshnick, S. R. *Antimicrob. Agents Chemother*., **2003**, *47*, 2712.

[95] Posner, G. H.; Oh, C. H. *J. Am. Chem. Soc.*, **1992**, *114*, 8328.

[96] Hong, Y. L.; Yang, Y. Z.; Meshnick, S. R. *Mol. Biochem. Parasitol.*, **1994**, *63*, 121.

[97] Rodriguez, M.; Claparols, C.; Robert, A.; Meunier, B. *Chembiochem.*, **2002**, *3*, 1147.

[98] Haynes, R. K.; Monti, D.; Taramelli, D.; Basilico, N.; Parapini, S.; Olliaro, P. *Antimicrob. Agents. Chemother.*, **2003**, *47*, 1175.

[99] Haynes, R. K.; Hung-On Pai, H.; Voerste, A. *Tetrahedron Lett.*, **1999**, *40*, 4715.

[100] Asawamahasakda, W.; Ittarat, I.; Pu, Y. M.; Ziffer, H.; Meshnick, S. R. *Antimicrob. Agents. Chemother.*, **1994**, *38*, 1854.

[101] Bhisutthibhan, J.; Pan, X. Q.; Hossler, P. A.; Walker, D. J.; Yowell, C. A.; Carlton, J.; Dame, J. B.; Meshnick, S. R. *J. Biol. Chem.*, **1998**, *273*, 16192.
[102] Sagara, Y.; Inesi, G. *J. Biol. Chem.*, **1991**, *266*, 13503.
[103] Plowe, C. V. *J. Exp. Biol.*, **2003**, *206*, 3745.
[104] Korsinczky, M.; Chen, N.; Kotecka, B.; Saul, A.; Rieckmann, K.; Cheng, Q. *Antimicrob. Agents Chemother.*, **2000**, *44*, 2100.
[105] Cohen, S. D.; Pumford, N. R.; Khairallah, E. A.; Boekelheide, K.; Pohl, L. R.; Amouzadeh, H. R.; Hinson, J. A. *Toxicol. Appl. Pharmacol.*, **1997**, *143*, 1.
[106] Nelson, S. D.; Pearson, P. G. *Annu. Rev. Pharmacol. Toxicol.*, **1990**, *30*, 169.
[107] Yang, Y. Z.; Asawamahasakda, W.; Meshnick, S. R. *Biochem. Pharmacol.*, **1993**, *46*, 336.
[108] Hansen, B. T.; Jones, J. A.; Mason, D. E.; Liebler, D. C. *Anal. Chem.*, **2001**, *73*, 1676.
[109] Liebler, D. C. *Environ. Health Perspect*., **2002**, *110* Suppl. 1, 3.
[110] Walker, D. J.; Pitsch, J. L.; Peng, M. M.; Robinson, B. L.; Peters, W.; Bhisutthibhan, J.; Meshnick, S. R. *Antimicrob. Agents. Chemother.*, **2000**, *44*, 344.
[111] Hussain, A.; Garnett, C.; Klein, M. G.; Tsai-Wu, J. J.; Schneider, M. F.; Inesi, G. *J. Biol. Chem.*, **1995**, *270*, 12140.
[112] Yu, M.; Lin, J.; Khadeer, M.; Yeh, Y.; Inesi, G.; Hussain, A. *Arch. Biochem. Biophys.*, **1999**, *362*, 225.
[113] Chawira, A. N.; Warhurst, D. C. *J. Trop. Med. Hyg*., **1987**, *90*, 1.
[114] Gupta, S.; Thapar, M. M.; Wernsdorfer, W. H.; Bjorkman, A. *Antimicrob. Agents Chemother.*, **2002**, *46*, 1510.
[115] Xiao, S. H.; Yao, J. M.; Utzinger, J.; Cai, Y.; Chollet, J.; Tanner, M. *Parasitol. Res.*, **2003**.
[116] Chou, A. C.; Chevli, R.; Fitch, C. D. *Biochemistry*, **1980**, *19*, 1543.
[117] Francis, S. E.; Sullivan, D. J.; Goldberg, D. E. *Ann. Rev. Microbiol.*, **1997**, *51*, 97.
[118] Dorn, A.; Vippagunta, S. R.; Matile, H.; Jaquet, C.; Vennerstrom, J. L.; Ridley, R. G. *Biochem. Pharmacol.*, **1998**, *55*, 727.
[119] Pagola, S.; Stephens, P. W.; Bohle, D. S.; Kosar, A. D.; Madsen, S. K. *Nature*, **2000** , *404*, 307.
[120] Sullivan, D. J. *Int. J. Parasitol.*, **2002**, *32*, 1645.
[121] Djimde, A.; Doumbo, O. K.; Cortese, J. F.; Kayentao, K.; Doumbo, S.; Diourte, Y.; Dicko, A.; Su, X. Z.; Nomura, T.; Fidock, D. A.; Wellems, T. E.; Plowe, C. V.; Coulibaly, D. *N. Engl. J. Med.*, **2001**, *344*, 257.
[122] Fidock, D. A.; Nomura, T.; Talley, A. K.; Cooper, R. A.; Dzekunov, S. M.; Ferdig, M. T.; Ursos, L. M.; bir Singh Sidhu, A.; Naude, B.; Deitsch, K. W.; Su, X.; Wootton, J. C.; Roepe, P. D.; Wellems, T. E. *Mol. Cell.*, **2000**, *6*, 861.
[123] Ginsburg, H.; Ward, S. A.; Bray, P. G. *Parasitol. Today*, **1999**, *15*, 357.
[124] Reed, M. B.; Saliba, K. J.; Caruana, S. R.; Kirk, K.; Cowman, A. F. *Nature*, **2000**, *403*, 906.
[125] Myrick, A.; Munasinghe, A.; Patankar, S.; Wirth, D. F. *Mol. Microbiol.*, **2003**, *49*, 671.
[126] Cowman, A. F.; Karcz, S.; Galatis, D.; Culvenor, J. G. *J. Cell. Biol.*, **1991**, *113*, 1033.
[127] Banumathy, G.; Singh, V.; Pavithra, S. R.; Tatu, U. *J. Biol. Chem.*, **2003**, *278*, 18336.
[128] Wilson, M.; DeRisi, J.; Kristensen, H. H.; Imboden, P.; Rane, S.; Brown, P. O.; Schoolnik, G. K. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 12833.
[129] Liebler, D. C.; Hansen, B. T.; Jones, J. A.; Badghisi, H.; Mason, D. E. *Adv. Protein. Chem.*, **2003**, *65*, 195.